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# Reduction of cortical amyloid β levels in guinea pig brain after systemic administration of physostigmine

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**Abstract**

Overproduction of the peptide amyloid β (Aβ) is thought to be a critical pathogenetic event in Alzheimer's disease (AD). Decreasing Aβ production may therefore slow or halt the progression of AD. In vitro work has indicated that cholinergic muscarinic receptor agonists may reduce cellular production of Aβ. Here we show that systemic administration of physostigmine, an acetylcholinesterase inhibitor, lowers Aβ levels in vivo. Guinea pigs treated for 10 days with s.c. physostigmine had levels of cortical AβN-40 and N-42 which were 57% and 72%, respectively, of those in control animals. Levels of cortical β-amyloid precursor protein were not significantly affected by drug treatment. These results suggest that cholinergic therapy may affect the course of AD by limiting Aβ accumulation. 

*Keywords*: Alzheimer's disease; Cholinergic; Therapy; Amyloid β; Physostigmine; Guinea pigs; Cerebral cortex

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. Two microscopic lesions, the senile or amyloid plaque and the neurofibrillary tangle, are hallmarks of the disease. Theories of AD pathogenesis to date have largely been concerned with the molecular mechanisms of their creation. A particularly convincing argument has been assembled supporting the hypothesis that abnormal metabolism of a single protein, the β-amyloid precursor protein (β-APP), is the cause of amyloid plaque formation. Overproduction of the peptide amyloid β (Aβ), a cleavage fragment of β-APP, appears to be a critical pathogenetic step which is central to all forms of AD [11].

Manipulating the pathways of β-APP metabolism is therefore one promising approach to AD therapy. β-APP is processed by one of two major pathways [19]. The first cleaves β-APP at the cell membrane, releasing the extracellular component of the molecule, sAPPα, into the interstitial fluid. Because this cleavage occurs in the middle of the Aβ fragment, it is also termed the non-amyloidogenic pathway since it precludes the accumulation of Aβ. In the alternative, amyloidogenic pathway, β-APP is cut into a variety of products, including sAPPβ and Aβ. Pharmacologic agents which decrease amyloidogenic processing of β-APP, thereby decreasing production of Aβ, might prevent amyloid plaque formation.

A substantial body of recent work has indicated that β-APP metabolism can be regulated by activation of muscarinic cholinergic receptors. In vitro studies have shown that muscarinic M1 and M3 agonists stimulate processing of β-APP via the non-amyloidogenic pathway, resulting in increased release of sAPPα and reduced Aβ production [4,5,7,16,17,21]. The present study was designed to determine whether systemic administration of physostigmine, an acetylcholinesterase inhibitor which increases the extracellular concentrations of brain acetylcholine [8], would decrease cerebral cortical β-APP and/or Aβ.

Guinea pigs were used for this study because their Aβ amino acid sequence is identical to that of humans [2]. Adult female Hartley guinea pigs were implanted under general anesthesia (ketamine 40 mg/kg and xylazine 5 mg/kg) with subcutaneous osmotic pumps (Alzet, Model 2ML2) containing physostigmine salicylate (9.1 mg/ml; Sigma) dissolved in sterile normal saline. The daily dosage for physostigmine-treated animals ($n = 7$) ranged from 2.41 to 2.84 mg/kg/day, depending on the weight of individual animals. Control animals ($n = 6$) were implanted with identical

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osmotic pumps containing only sterile normal saline. After 10 days, the animals were euthanized with an overdose of sodium pentobarbital. After brain removal the cerebral hemispheres were isolated, sliced in the coronal plane into 0.4 cm segments and frozen on slabs of dry ice.

For dot-blot analysis of β-APP, cortical samples were dissected and homogenized in 20 mM Tris buffer, (pH 7.5), with 2 mM EDTA, 2 mM EGTA, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 100 μg/ml PMSF (all chemicals obtained from Sigma). After protein determinations (micro-Lowry), a series of graded dilutions (1, 2.5, 5.0, 7.5 and 10.0 μg protein/100 μl) were prepared from each animal (final volume 100 μl/well) and filtered in a 96 well Bio-dot apparatus (Biorad) on PVDF transfer membrane (NEN Research Products). After blotting, the membranes were dried and incubated overnight in blocking buffer (TBS, pH 8.0, with 0.05% Tween, 2% skim milk and 0.5% normal goat serum; all dilutions and wash steps hereafter also used this buffer), followed by overnight incubation with 22C11 (directed at the N terminus of β-APP, Boehringer-Mannheim) or ALZ-90 (directed at amino acids 511–608 of β-APP, Boehringer-Mannheim) monoclonal antibodies, diluted 1:50. After washing, the membranes were incubated for 2 h at room temperature with alkaline phosphatase-coupled anti-mouse IgG secondary antibody (Sigma) diluted 1:3000. Following a final wash, the colored reaction product was created using the substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine. Quantification was performed by scanning the blots with a video camera and analyzing with the NIH Image program. Plotting of the data showed that the optical density of the reaction product had a linear relationship with protein concentration over the range of homogenate dilutions employed. Interpolation was used to obtain the midpoint, for each animal, of the best-fit line between the points representing the values from each of the five dilutions. This value was used in subsequent statistical calculations.

Quantification of Aβ was performed as previously described [13]. Soluble and insoluble Aβ, defined as the fractions solubilized by aqueous buffer or formic acid, respectively, were determined separately, as evidence from human [14,20] and animal studies [13] indicate that both may play potentially important roles in amyloidogenesis and toxicity. Cortical samples were homogenized in five volumes of 50 mM Tris–hydroxymethyl aminomethane-HCl, (pH 7.5) buffer using a Dounce glass homogenizer. The protein concentration of each specimen was determined using the BCA method (Pierce). Once the volumes were adjusted for protein concentration, the specimens were centrifuged at 100 000 × *g* for 30 min at 4°C. The supernatants were directly submitted to europium immunoassay for quantification of soluble AβN-40 and AβN-42, using the end-specific antibodies R163 and R165, respectively. The pellets were homogenized in five volumes of 80% glass distilled formic acid and centrifuged at 100 000 × *g* for 30 min at 4°C. Five hundred microlitres of the supernatant were chromatographically separated by size exclusion on a Superose 12 column. The 4–5 kDa fractions were collected, the acid removed by vacuum centrifugation, resuspended and neutralized. Aliquots of 100 μl were plated on microtiter plates for europium immunoassay determination of insoluble AβN-40 and AβN-42 as for the soluble fraction.

For β-APP and Aβ data, the physostigmine and control groups were compared using unpaired, two-tailed *t*-tests. The significance level for all comparisons was set at 0.05.

The mean optical densities of the reaction product for β-APP in physostigmine-treated animals did not differ significantly from those for saline-treated animals, using either the 22C11 or ALZ-90 antibodies (data not shown). Physostigmine had no statistically significant effect on the cortical concentrations of soluble AβN-40 or soluble AβN-42 (Table 1) but significantly lowered the concentration of insoluble Aβ1–40 (Table 1), with respect to that of the saline-treated control animals ($P = 0.035$). Mean insoluble AβN-40 concentrations were reduced to 57% of those in saline-treated animals. The same trend was observed for insoluble AβN-42, with physostigmine-treated animals having a reduction to 72% of control levels.

Cholinergic replacement has long been the only effective pharmacological treatment for Alzheimer's disease, but it has generally been thought to be useful only for ameliorating the cognitive symptoms. The discovery that muscarinic receptor activation could regulate β-APP processing [17] made it theoretically possible that cholinergic therapy could also be used to slow the disease process, by decreasing Aβ production. Cell culture studies have shown that M1 and M3 muscarinic agonists increase the secretion of sAPPα into the culture medium [4,16] and decrease production of Aβ [7,21]. Studies of rat brain slices have yielded similar results, in that both muscarinic agonists [5,16] and acetylcholinesterase inhibitors [15] cause increased sAPPα secretion.

Table 1
Mean cortical concentrations (pg/mg protein) of soluble and insoluble Aβ N-40 and N-42 in physostigmine-treated and control groups[a]

| | Soluble Aβ N-40 | Soluble Aβ N-42 | Insoluble Aβ N-40 | Insoluble Aβ N-42 |
|---|---|---|---|---|
| Control | 3.65 (0.749) | 81.58 (7.51) | 78.55 (13.54) | 17.70 (7.33) |
| Physostigmine | 3.40 (0.921) | 93.46 (10.90) | 38.24 (27.36)* | 12.78 (3.76) |

[a] Figures in parentheses represent SEM. *$P = 0.035$.

In vivo studies are needed to determine whether systemic cholinergic therapy can affect β-APP processing in the brain, and, in particular, whether brain Aβ concentrations can be lowered. A small number of studies have already addressed this question. It has recently been shown that systemic administration of an M1-selective muscarinic agonist reduces cerebrospinal fluid (CSF) levels of total Aβ in humans with AD [18]. We have found that M1-selective muscarinic agonists reduce CSF levels of both Aβ1–40 and Aβ1–42 in rabbits [1]. In addition, rats treated with acetylcholinesterase inhibitors had decreased cerebrospinal fluid levels of sAPPβ, an amyloidogenic pathway metabolite [9].

The results indicate that systemic physostigmine administration lowers insoluble cortical AβN–40 concentrations. A trend for the same effect was seen with Aβ N-42. Drug-treated animals did not differ significantly from control animals in the amounts of cortical β-APP, indicating that the changes seen in Aβ concentrations were probably due to altered β-APP processing, rather than simple downregulation of β-APP.

Only insoluble Aβ species were affected by the drug treatments. The reason for this is unknown. There has been considerable discussion in the literature as to the relative importance of soluble and insoluble Aβ in the pathogenesis of AD. While much evidence indicates that only the insoluble, β-pleated sheet form of Aβ is neurotoxic or inflammation-provoking, it is clear that only diffusely-distributed soluble molecules would be able to react widely with cellular receptors mediating such effects. And while it is primarily the accumulation of insoluble Aβ that distinguishes AD from normal aging [14,20], it has seemed logical that insoluble Aβ is created as a result of concentration-dependent precipitation of soluble forms.

These results strengthen the evidence that cholinergic pharmacotherapy may have more than a symptomatic role in AD management. By reducing insoluble cortical Aβ concentrations, acetylcholinesterase inhibitors and/or M1/M3 muscarinic agonists may be able to slow amyloid deposition and hence disease progression. Clinical studies support this hypothesis, as Cognex (tacrine, THA), Aricept (donepezil) and Exelon (rivastigmine), which are acetylcholinesterase inhibitors already in use for AD therapy, have all been reported to slow the rate of cognitive and/or functional decline [3,6,10,12]. As Aβ deposition begins years before clinical diagnosis, it is possible that presymptomatic therapy could have even greater effects.

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